Oculodentodigital dysplasia connexin43 mutations result in non-functional connexin hemichannels and gap junctions in C6 glioma cells

Albert Lai1,2,*, Dung-Nghi Le1,2, William A. Paznekas3, Wes D. Gifford1,2, Ethylin Wang Jabs3,4 and Andrew C. Charles1

1Department of Neurology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
2The Henry E Singleton Brain Cancer Research Program, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
3Institute of Genetic Medicine, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA
4Departments of Medicine and Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

*Author for correspondence (email: albertlai@mednet.ucla.edu)

Accepted 31 October 2005
Journal of Cell Science 119, 532-541 Published by The Company of Biologists 2006
doi:10.1242/jcs.02770

Summary
Oculodentodigital dysplasia (ODDD) is a rare developmental disorder characterized by craniofacial and limb abnormalities. Over 35 separate mutations in human connexin43 (Cx43) causing ODDD have been identified. Several mutations are also associated with central nervous system involvement, including white-matter changes detected by magnetic resonance imaging. As Cx43 is abundantly expressed in astrocytes, we hypothesized that the mutant Cx43 proteins that produce neurological dysfunction have abnormal functional characteristics in astrocytes. To understand how ODDD-associated mutations affect Cx43 signaling in cells of glial origin, we conducted studies in rat C6 glioma cells, a communication-deficient glial cell line that expresses low levels of Cx43. We generated stable cell lines expressing enhanced yellow fluorescent protein (eYFP)-tagged human Cx43 constructs encoding wild-type and six eYFP-tagged mutant Cx43 mutants: Y17S, G21R, A40V, F52dup, L90V and I130T. Of these, Y17S, L90V and I130T are associated with neurological abnormalities. We found that all mutants could be detected on the cell surface. Y17S, G21R, A40V, L90V and I130T formed triton-resistant plaques representing gap junctions, although the relative ability to form plaques was decreased in these mutants compared with the wild type. F52dup formed dramatically reduced numbers of plaques. Propidium iodide uptake experiments demonstrated that all mutants were associated with reduced connexin hemichannel function compared with wild type. Scrape-loading experiments performed on the same stable cell lines showed reduced gap junctional dye transfer in all mutants compared with the wild type. These studies demonstrated that ODDD-associated Cx43 mutations result in non-functional connexin hemichannels and gap junction functions in a glial cell line regardless of whether the particular mutant is associated with neurological dysfunction.

Key words: Connexin43, Oculodentodigital dysplasia (ODDD), Hemichannel, Gap junction, Glia

Introduction
Connexins oligomerize to form gap junctions (Nicholson, 2003; Saez et al., 2003). Gap junctions allow direct intercellular communication between adjacent cells via transfer of ions and molecules less than 1 kDa in size. Formation of a gap junctional channel requires docking of two connexons, also known as hemichannels. The existence of connexin hemichannels has been previously viewed as a transient intermediate step in gap junction assembly. Recent studies suggest that connexin hemichannels may function independently to mediate physiological release of signaling molecules such as ATP, glutamate, PGE2 and NAD into the extracellular space, thereby enabling paracrine stimulation of neighboring cells (Bennett et al., 2003; Goodenough and Paul, 2003; Jiang and Cherian, 2003).

Cx43 is a connexin family member that is expressed throughout human tissues, including the central nervous system, heart and bone (Wei et al., 2004). Within the central nervous system, Cx43 is the primary gap junction constituent in astrocytes (Theis et al., 2004). Recently, the importance of Cx43 in promoting normal development has been emphasized by the discovery that ODDD, a rare pleiotropic developmental disorder that is usually inherited in an autosomal dominant fashion, is caused by mutations in Cx43 (Paznekas et al., 2003). Thus far, over 35 distinct missense mutations have been published that involve most domains of the protein. In addition, a mutation in the C-terminal cytoplasmic tail (C260fsX307) that results in a truncated protein with 46 incorrect amino acids has also been described (Kjaer et al., 2004; Paznekas et al., 2003; Pizzuti et al., 2004; Richardson et al., 2004; Vitiello et al., 2005).

The pleiotropic phenotype can be presumably explained by the presence of Cx43 in multiple tissue types throughout the body. The mutations in Cx43 that are associated with ODDD parallel several disease-causing mutations in other connexin genes, such as Cx26 and Cx32. A large array of different...
mutations that can be found in nearly every domain of these proteins can cause phenotypic variations. For example, several Cx43 mutations appear to cause neurological dysfunction such as spasticity and neurogenic bladder disturbances that can be accompanied by prominent white-matter changes detectable by brain MRI (Loddenkemper et al., 2002). The ability of mutant Cx43 to cause white-matter dysfunction is not unexpected given the abundance of Cx43 in astrocytes, reinforcing the importance of Cx43 in maintaining normal astrocyte function. Although each mutation may have a cell-type specific effect on Cx43 function, the mechanisms that enable some mutations to cause neurological dysfunction remain unclear. This issue is further complicated by the presence of interfamilial phenotypic variability. Interestingly, except for G21R, which was associated with a cardiac atrial-septal defect, other mutations do not appear to cause cardiac abnormalities, despite the abundance of Cx43 in cardiac tissue (Paznekas et al., 2003; Shibayama et al., 2005).

In this study, we have analyzed the ability to form functional hemichannels and gap junctional plaques for six (Y17S, G21R, A40V, F52dup, L90V and I130T) ODDD-associated Cx43 mutants expressed as eYFP-tagged fusion proteins in rat C6 glioma cells. The rationale for selecting C6 cells was that they have been used as a model for astrocytes and the possibility that they could enable the determination of differences between mutations causing neurological dysfunction from those that do not. In addition, C6 cells have been particularly useful in the study of Cx43 signaling because they are communication deficient.

Results

Stable expression of wild-type and mutant Cx43-eYFP in C6 cells

To examine the localization and function of wild-type and ODDD-associated mutant Cx43 proteins in glial cells, eYFP-tagged Cx43 cDNA constructs were stably expressed in rat C6 glioma cells using the BH-RCAS retroviral expression system (Hughes et al., 1990; Lai et al., 1998; Odorizzi et al., 1996). C6 cells have low but detectable levels of endogenous Cx43 expression (Ozog et al., 2002) and have negligible levels of gap junctional intercellular coupling (GJIC) and connexin-hemichannel mediated transmembrane flux (Stout et al., 2002; Zhu et al., 1991). The eYFP-tagged Cx43 constructs consist of the Cx43 sequence joined at its C terminus with eYFP by a short linker segment consisting of eight amino acids; the locations of the six mutations expressed and analyzed are indicated in Fig. 1. GFP-tagged connexins, including Cx43, have been used extensively for trafficking analysis and GJIC function (Bukauskas et al., 2000; Jordan et al., 1999; Roscoe et al., 2005; Seki et al., 2004; Shibayama et al., 2005). Fusion of the tag to the C-terminal domain of Cx43 as opposed to the N terminus does not interfere with GJIC or hemichannel function (Contreras et al., 2003). GFP-tagging is not without its consequences, as evidenced by one observation that larger plaques are formed from GFP-tagged Cx43 expressed in HeLa cells (Hunter et al., 2003). The authors hypothesized that the GFP tag impairs interactions between the Cx43 C-terminal domain and cellular proteins. One such protein shown to interact with the Cx43 C-terminal domain is ZO-1 (Toyofuku et al., 2001).

Using an anti-GFP polyclonal antibody, a prominent band representing the full-length wild-type Cx43-eYFP fusion protein was detected at ~70 kDa and was not detected in C6R (C6 cells expressing the Tva receptor, see Materials and Methods) cells (Fig. 2A). A minor band that co-migrates at ~30 kDa with the control eGFP band was seen at variable levels with cell lines expressing other mutants (data not shown). This protein species could represent either independent translation of the eYFP-coding sequence or an intracellular degradation product. A similar band was found in western blotting analysis of Cx36-eGFP fusion proteins, but has not been reported in Cx43-eGFP fusions (Zoidl et al., 2002).

Probing with a rabbit polyclonal anti-Cx43 antibody, the ~70 kDa band was again detected in cells expressing wild-type Cx43-eYFP and in cells expressing mutant Cx43-eYFP fusion proteins but not C6 cells or uninfected C6R cells (Fig. 2B). We confirmed that equivalent amounts of total protein were loaded for each cell line by probing for actin (Fig. 2B). Except for L90V, the amount of Cx43-eYFP fusion protein is similar or greater (Y17S, F52dup, 130T) than wild type. More quantitative analysis of surface expression is described below (Fig. 2E). In all cell lines, there was also a band migrating at ~40 kDa detectable in C6 and uninfected C6R cells, representing endogenous Cx43 expression. However, G21R and I30T cell lines were found to have higher levels of apparent endogenous Cx43 expression. This raised that possibility that either this increased band represents degradation from the fusion protein or that these mutant fusion proteins somehow increase expression or reduce degradation of the endogenous pool of Cx43. There was also another band migrating at ~50 kDa seen in lysates from Y17S, I130T, and most strikingly from A40V. This band may represent a degradation product that was differentially generated amongst these mutants. Interestingly, Y17S and I130T but not A40V were associated
with neurological abnormalities. The possibility arises that such 'extra' bands, detected with either anti-Cx43 or -GFP antibodies, could interfere with trafficking, assembly and distribution of full-length Cx43-eYFP proteins.

Localization of wild-type and mutant Cx43-eYFP by confocal microscopy

To determine the subcellular localization of Cx43-eYFP constructs, we used confocal fluorescence microscopy to detect eYFP fluorescence in live cells. As expected, wild-type Cx43-eYFP was found in punctate structures located at cell-cell junctions that are likely to represent gap junction plaques (Fig. 3B); no fluorescence was detected in uninfected C6R cells (Fig. 3A). All mutants appeared to form such punctate structures with reduced abundance compared with wild type. Mutants L90V (Fig. 3G) and I130T (Fig. 3H) showed a slightly reduced abundance of puncta while mutants Y17S (Fig. 3C), G21R (Fig. 3D) and A40V (Fig. 3E) formed significantly less puncta than wild type. F52dup appeared to be localized at the cell membrane, with only occasional formation of punctate structures at the cell surface (Fig. 3F). Slightly increased numbers of puncta could be induced by longer periods of culture on glass coverslips, suggesting that F52dup does not completely abolish the ability to form gap junctions (data not shown). It was difficult to determine whether this reflects a true difference in ability to form these plaque structures or simply differences in expression level between the cell lines or plating density and confluence. Overall, these results indicated that all of the mutations tested inhibit the ability to form punctate structures at the cell surface to varying degrees. Interpretation of these results must take into consideration the presence of endogenous Cx43 found in C6 cells, which may enable mutant connexins to be inserted at the cell surface (Essenfelder et al., 2004).
Surface expression of wild-type and mutant Cx43-eYFP
To confirm biochemically that Cx43-eYFP constructs could be found on the cell surface, we biotinylated the cells at 4°C to selectively label cell-surface proteins and isolated biotinylated proteins with streptavidin-agarose beads. Although it is unclear whether non-junctional and junctional Cx43 are labeled and recovered by this method with the same efficiency, we have found that 15-20% of the total surface fraction of Cx43-eYFP isolated in this way is Triton X-100 insoluble, indicating that at least some if not all junctional Cx43 is recovered (data not shown).

Western blot analysis of these samples showed that the ~70 kDa band was detected using both anti-GFP and anti-Cx43 antibodies from cells expressing wild-type Cx43-eYFP (Fig. 2C,D). The absence of surface detection of the cytoplasmic eGFP demonstrated the specificity of this procedure for surface proteins (compare eGFP lane in Fig. 2A with 2C). Likewise, the absence of actin recovered from streptavidin-agarose beads provided additional confirmation of the selectivity of this procedure (compare Fig. 2B with 2D).

Comparison of the relative intensities of the fusion protein between the wild type and the mutants showed that all mutants, except for L90V, were expressed on the surface at similar or greater levels than the wild type. Comparison of the relative intensities of the presumptive endogenous Cx43 band migrating just above 40 kDa showed that all mutant cell lines have similar or increased surface expression (G21R and I130T). As was the case for the whole lysates, the exact nature of this effect is unclear but explanations include the possibilities that these mutants substantially increased trafficking and insertion of endogenous Cx43 on the cell surface, inhibited degradation of surface Cx43 or produced a degradation product co-migrating at 40 kDa. Qualitative comparison of the ratio of surface wild-type Cx43-eYFP to total wild-type Cx43-eYFP with the ratio of surface endogenous Cx43 from C6 or C6R to total endogenous Cx43 from C6 or C6R (Fig. 2B,D) may provide an explanation for the functional silence of endogenous Cx43 in C6 cells. These data suggested that the endogenous Cx43 has significantly decreased insertion on the cell surface.

As functional activity of Cx43-eYFP fusion proteins was likely to be dependent on surface expression, we performed quantitative analysis of surface expression by measuring amount Cx43-eYFP fusion protein bound to the streptavidin-agarose beads in a fluorescence microplate reader for two clonal cell lines per mutant (Fig. 2E). These results confirmed the qualitative results shown from the western blot analysis (Fig. 2D) and demonstrated that each mutant except for L90V was expressed on the surface at greater levels than wild type. In the case of A40V, F52dup and one clone of I130T, nearly fourfold more surface expression per μg protein was found relative to wild type.

Assembly of wild-type Cx43 and mutant Cx43-eYFP into Triton-resistant plaques at cell junctions
To provide further evidence that these punctate structures were gap junctional plaques, we examined whether such structures were resistant to extraction by 1% Triton X-100 in live cell monolayers (Thomas et al., 2004). This procedure was based on the property of gap junctional plaques to acquire resistance to Triton X-100 extraction after assembly (Musil and Goodenough, 1991).

![Fig. 3. Subcellular localization of Cx43-eYFP fusion proteins reveals formation of punctate structures at cell-cell junctions.](image)

Immunofluorescence microscopy of live cells growing on glass coverslips was performed on a custom laser-scanning confocal microscope equipped with a blue diode laser (475 nm) using a 63×1.3 objective.

These data are representative of separate experiments on two cell lines for each construct. (A) Uninfected C6R was included as a control demonstrating lack of fluorescence signal. Mutants L90V (G) and I130T (H) showed a slightly reduced abundance of puncta, while mutants Y17S (C), G21R (D) and A40V (E) formed significantly fewer puncta than wild type. F52dup appeared to be localized at the cell membrane with only occasional formation of punctate structures at the cell surface (F). Scale bar, 10 μm.
Live cell monolayers were first imaged in HBSS (Fig. 4A,C,E,G,I,K,M). The identical field was then imaged after incubation with HBSS containing 1% Triton for 15 minutes at room temperature (Fig. 4B,D,F,H,J,L,N). We found that many of the punctate structures in the wild type and mutants forming such structures were Triton resistant, indicating that these structures are gap junctional plaques. Interestingly, F52dup also demonstrated Triton-resistant fluorescence that was not associated with the large punctate structures, suggesting that F52dup mutant connexins formed Triton-resistant structures without being able to aggregate into larger plaques (Fig. 4I,J).

**Ca**\(^{2+}\) withdrawal stimulated PI uptake by cells expressing wild-type Cx43-eYFP but not by cells expressing mutant Cx43-eYFP fusion proteins

To determine whether mutant Cx43-eYFP fusion proteins form functional hemichannels in C6 cells when compared with wild-type Cx43-eYFP, we performed quantitative hemichannel uptake assays on live cell monolayers using the low-molecular weight dye propidium iodide (PI; 668 Da) to detect the presence of functional hemichannels. Low-molecular weight fluorescent dyes that bind to DNA such as PI or ethidium bromide have been used to assay hemichannel activity (Contreras et al., 2002; Kondo et al., 2000). Under normal ionic conditions, connexin hemichannels are largely blocked by extracellular divalent cations. Removal of extracellular Ca\(^{2+}\) opens the channels (Stout et al., 2002; Thimm et al., 2005). In a representative experiment using confluent monolayers (Fig. 4A,D), we compared uptake of PI between control C6R cells expressing eCFP (Fig. 5B,C) with wild type (Fig. 5E,F) in regular HBSS (with Ca\(^{2+}\) and Mg\(^{2+}\)) (Fig. 5B,E) and HBSS without Ca\(^{2+}\) and Mg\(^{2+}\) (Fig. 5C,F) after 15 minutes. In control C6R cells, no uptake was seen in regular HBSS. Occasional bright nuclei appeared that probably represent dead cells (Fig. 5B). Incubation of these cells with HBSS lacking Ca\(^{2+}\) and Mg\(^{2+}\) resulted in a minimal increase in background in all cells representing either non-specific uptake or uptake via endogenous Cx43 and a small percentage of extremely bright cells that could represent additional cell death during this incubation condition (Fig. 5C). These bright cells were excluded from the quantitative analysis reported (Fig. 5G). By comparison, incubation of C6 cells expressing wild-type with regular HBSS caused low levels of uptake in virtually every cell (Fig. 5E), whereas incubation in HBSS lacking Ca\(^{2+}\) showed a large increase in uptake (Fig. 5F).

Quantitation of these results and those of experiments on cells expressing mutants was achieved using ImageJ software by averaging the average intensities of 40 cells per field (Fig. 5G). None of the mutants had uptake in regular HBSS that was greater than the wild type, indicating that none of the mutations resulted in constitutively active hemichannels. Similarly, none of the mutants had significant activity greater than control cells (C6 or C6R) upon incubation in HBSS lacking Ca\(^{2+}\), indicating that these mutations prevented formation of hemichannels or impaired function of formed hemichannels. The slight increased uptake of wild type compared with C6R or C6 upon incubation in HBSS with Ca\(^{2+}\) may have indicated either constitutive uptake through wild-type hemichannels or transient activation stimulated by media changes.

**Cells expressing mutant Cx43-eYFP lacked gap junctional intercellular communication (GJIC), as measured by scrape-loading**

To determine whether mutant Cx43-eYFP could mediate gap junctional transfer of low molecular weight dyes, we performed scrape-loading experiments with sulforhodamine B (559 Da) on cell monolayers expressing wild-type and mutant Cx43-eYFP. We used this dye because its fluorescence spectrum is different from that of eYFP, enabling easier interpretation of results. Although the dye is not fixable, we were able to overcome the problem of dye leakage by immediately photographing scrape-loaded monolayers at a set time after loading. In a representative experiment comparing the extent of scrape-loaded dye transfer between eCFP-expressing C6R control cells (Fig. 6A,B) and wild-type expressing cells (Fig. 6C,D), we found that cells expressing wild-type Cx43-eYFP (Fig. 6C) enable transfer of dye 4-6 cell layers beyond the initially loaded cells, whereas

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**Fig. 4.** Punctate structures are resistant to Triton X-100 extraction. Confocal immunofluorescence microscopy was performed on live C6R cell monolayers expressing wild-type and mutant Cx43-eYFP constructs, as indicated after incubation in HBSS (A,C,E,G,I,K,M) and after incubation for 15 minutes in HBSS containing 1% Triton X-100 (B,D,F,H,J,L,N) on the same field. Significant numbers of puncta remained after Triton X-100 extraction for the wild type and the various mutants. Although F52dup did not form plaque structures, some of the cell surface signal was resistant to extraction. Real-time monitoring of Triton X-100 extraction revealed that signal extracted was nearly instantaneously after addition of Triton. Overlays of images cannot be created because of an apparent mild deformation of cell architecture by Triton X-100 in non-fixed cells. Scale bar, 10 μm.
transfer to only 0-1 cell layers was detected for control C6 cells expressing eCFP. To determine which cells represented cells wounded by the scrape loading, we performed experiments with rhodamine-conjugated dextran (10 kDa), a gap junction-impermeable dye conjugate, confirming that these cells were confined to the brightest cells adjacent to the scrape (Fig. 6B,D). Scrape-loading analysis of dye transfer in a representative cell line expressing each mutant demonstrated that all mutants showed no appreciable dye transfer as no fluorescence is detected beyond the initially wounded cells adjacent to the scrape (Fig. 6G-L).

**Discussion**

To characterize the functional consequences of ODDD-associated Cx43 mutations in glial cells, we stably expressed wild-type and six mutant (Y17S, G21R, A40V, F52dup, L90V and I130T) Cx43-eYFP proteins in rat C6 glioma cells using a retroviral expression system. Using confocal microscopy and surface biotinylation, we found that all six mutant Cx43-eYFP were localized to the cell surface, with all mutants except F52dup forming significant numbers of Triton X-100 resistant puncta at cell-cell junctions, indicating the presence of gap junctional plaques. Qualitatively, all mutants appeared to form puncta less efficiently than did the wild type, with most significant decreases in plaque abundance seen for Y17S, G21R and A40V. F52dup formed Triton X-100-resistant puncta at very low frequency with slightly increasing numbers detected at high confluency and prolonged culture times on the glass coverslips. This suggested that F52dup inhibited but did not completely abolish the formation of gap junctional plaques. It is possible that this effect occurs because of a conformational change in the area near C54 preventing disulfide bonding required for gap junctional formation (Bao et al., 2004; Foote et al., 1998). If so, one prediction was that F52dup mutant Cx43 are present on the cell surface predominantly as hemichannels. However, functional analysis

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Fig. 5. Mutant Cx43-eYFP do not form functional hemichannels as assessed by propidium iodide uptake. C6 cells, C6R control cells and clonal C6R cell lines expressing Cx43-eYFP constructs were analyzed for hemichannel function by determining the level of propidium iodide uptake after opening hemichannels by incubation of cell monolayers in 0 Ca\(^{2+}\). Stable expression of each construct in every cell was determined by detecting eCFP or eYFP fluorescence (A,D). A representative experiment showing PI uptake for control C6R (eCFP) (B,C) and wild-type Cx43-eYFP (E,F) after incubation in HBSS (B,E) with Ca\(^{2+}\) or HBSS (C,F) without Ca\(^{2+}\) containing PI for 15 minutes. Cells were fixed and uptake was visualized on a Nikon upright fluorescent microscope using rhodamine filter settings using a 20× objective. Digital photographs were quantitated using ImageJ software by averaging randomly selected average cell intensities for 40 cells per coverslip. (G) Quantitation of C6, C6R, wild type and each mutant is represented for HBSS with Ca\(^{2+}\) (white) and HBSS without Ca\(^{2+}\) (hatched). Data are expressed as mean±s.e.m. of several experiments on at least two cell lines for each mutant. Scale bar, 100 μm.
did not demonstrate increased hemichannel activity for F52dup, indicating that F52dup may also disrupt hemichannel function. Functional analysis of the remaining mutants demonstrated that each showed impaired hemichannel and GJIC function. This impaired function was observed in mutants with and without reported associated neurological dysfunction. From our results, it is difficult to conclude whether mutants inhibit hemichannel formation or function of formed hemichannels.

Thus far, three studies have been reported on localization, assembly and gap junction analysis of ODDD-associated Cx43 mutations (Roscoe et al., 2005; Seki et al., 2004; Shibayama et al., 2005). In the study by Roscoe et al., fluorescent eGFP-tagged G21R and G138R were expressed in HeLa cells (a cell type that lacks Cx43 expression and is deficient in GJIC) and NRK cells (a cell type that expresses Cx43 and has GJIC) (Roscoe et al., 2005). Although no significant trafficking or assembly defects were detected in those mutant Cx43 expressed in HeLa cells, both mutant Cx43 proteins formed non-functional gap junctions. When the mutants were expressed at levels several fold higher than the wild-type expression in NRK cells, both mutants were found to assert dominant-negative effects on GJIC. Hemichannel function of these mutants was not assessed in either cell type. Similar to these results in HeLa cells, our results with G21R show that non-functional gap junctions are formed in C6 cells. Seki et al. performed functional assays on N2A cells expressing I130T, K134E, G138R either tagged or untagged with eGFP, and found that these mutants had preserved ability to form plaques but had decreased function as measured by electrical coupling (Seki et al., 2004). Occasional coupling was found for cells expressing I130T. Our results on C6 cells expressing I130T were similar, although the scrape-loading assay probably lacks sufficient sensitivity to detect this low level of coupling. Very recently, Shibayama et al. reported on gap junctional formation and function of L90V, Y17S, A40V, I130T, G21R, K134E, F52dup and R202H mutant Cx43, tagged with eYFP and transiently expressed in HeLa or N2A cells (Shibayama et al., 2005). They found that all mutants had reduced levels of plaques, with F52dup and R202H not forming any plaques at all. F52dup was found to have ER localization. We also observed lack of plaque formation for F52dup, although in contrast to the finding of ER localization, we observed increased surface expression in our system. In the same report, junctional conductances between homotypically paired N2A cells showed dramatic decrease for all mutants, although current was measured for I130T, K134E and L90V. These results are similar to our data where we found decreased function for all mutants tested.
Mutations in connexin32 (Cx32) cause X-linked Charcot-Marie-Tooth disease (CMTX). Over 240 mutations have been identified that are located in every domain of Cx32 (Yum et al., 2002). Although only one ODDD mutation altering the C-terminal cytoplasmic tail of Cx43 has been identified, the same domain of Cx32 is the site of over 10 mutations. The trafficking of over 50 CMTX Cx32 mutant proteins have been analyzed in mammalian cells allowing comparison with our results. The mutant Cx32 A39V, A39P and A40V, coinciding with the A40V ODDD Cx43 mutant located in the first transmembrane region, were found to be retained in the ER (A39V, A39P) or retained in the Golgi in HeLa cells (Kleopa et al., 2002; Yum et al., 2002). In the case of A39V, the ER localization was also confirmed in Schwann cells expressing negligible levels of Cx32. One possible explanation for the different results of others and ours is based on our western blot analysis showing that control C6 cells express a low level of endogenous Cx43 (Fig. 2B). Although this level was not associated with GJIC or evidence of functional hemichannels, it is possible that endogenous Cx43 could mask the appearance of an intracellularly retained mutant Cx43 by chaperoning it to the cell surface (Essenfelder et al., 2004). As ODDD is for the most part an autosomal dominant disorder, most individuals with this condition will have a mutant allele and a wild-type allele, suggesting that the presence of endogenous Cx43 and mutant Cx43 in our C6 model may be a closer approximation than HeLa cells. Other Cx32 mutations (G21D and L90V) coinciding with the ODDD mutations examined in this study have been reported but no trafficking analysis has yet been reported. Disease-associated mutations in Cx26 coinciding with A40V (Montgomery et al., 2004) and L90V (Lim et al., 2003) have also been detected, but no functional or trafficking studies have been reported.

Thus far, all ODDD mutants analyzed by us have functional inactivity, presumably through impaired trafficking to the cell surface, impaired gap junctional assembly, or impaired gap junctional function. This is similar to the effects of different disease mutations on Cx32 and Cx26 function (Bruzzone et al., 2003; D’Andrea et al., 2002). However, other ODDD mutations may have preserved function or even gain-of-function. For example, the G11R and A88V Cx30 mutants are constitutively open, and also have GJ function (Essenfelder et al., 2004). Similarly, the D178Y Cx32 mutants result in deregulation of hemichannels by Ca2+ (Gomez-Hernandez et al., 2003).

As all of the ODDD mutations showed impaired gap junction and hemichannel function, it is not clear why the phenotype of the individuals with different mutations is variable. Regarding the neurological phenotype, the white matter changes on MRI occurred in only a subset of individuals. It is possible that some individuals who did not initially have these changes could develop them later. It is also possible that other factors are involved in the phenotypic variability. In some individuals and in some organ systems, there may be sufficient duplication of function by other connexins to overcome impaired Cx43 function. Another possibility is that the phenotypic variation between individuals with different mutations may fall in the spectrum of intrafamilial variation. Alternatively, some phenotypic differences may not actually exist if more detailed and lengthy follow-up is performed. For example, it is possible that all individuals will eventually develop neurological symptoms if followed long enough. Analogously, the heart is another organ with abundant expression of Cx43 and, like the central nervous system, does not appear to be uniformly involved in all individuals with ODDD. In fact, only one mutation, G21R, is associated with a heart defect (atrial-septal defect) (Paznekas et al., 2003; Shibayama et al., 2005).

Nonetheless, the lack of function of all mutant ODDD tested thus far despite differences in phenotype implies that other functional assays other than dye uptake and transfer may be required to discern differences between mutants with different phenotypes or severity. For example, the V84L Cx26 mutant has preserved ability to transfer Lucifer Yellow compared with wild-type Cx26. By contrast, the authors provide data showing that this mutant appears to have decreased ability to transfer PI6 compared with wild-type Cx26, leading to their interpretation that mutations have potential to change the selectivity of the channel (Beltramello et al., 2005). More detailed studies will be required to elucidate how Cx43 mutations cause ODDD.

Materials and Methods

Cell culture

C6 glial cells and chicken DF-1 fibroblasts were maintained in DMEM/F12 (Mediatech, Herndon, VA) containing 10% fetal bovine serum (HyClone, Logan, UT) and 100 U ml−1 penicillin and 100 µg ml−1 streptomycin in a 5% CO2 humidified atmosphere. DF-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). C6 cells were a gift from Dr C. Naus (University of British Columbia, Vancouver, Canada).

Generation of mutant Cx43 constructs

A product consisting of 201 bp of the 5′ untranslated region and the coding region of the wild-type human Cx43 (GJA1) sequence was amplified and cloned using adapter primers (forward primer with the underlined HindIII recognition site 5′-ACGAAGCTTATCGAGTATCAG-3′ and reverse primer with the underlined XmaI recognition site without a stop codon 5′-CTTCCGGGAGATCTCCAGGT- CATCAGGCC-3′) into the vector pEYFP-N1 (BD Biosciences Clontech, Palo Alto, CA) to encode and translate Cx43 with a C-terminal-linked eYFP fluorescent tag. Two-stage mutagenesis was performed using the above wild-type plasmid as the template to introduce six different ODDD related mutations into the Cx43 sequence. In brief, an upstream forward primer and a mutagenic reverse primer were used to amplify a 5′ product carrying the mutation and an overlapping 3′ product was amplified with a forward mutagenic primer (complement of the mutagenic reverse primer) and a downstream reverse primer. The 5′ and 3′ Cx43 amplification products were combined and amplified with the HindIII forward and XmnI reverse adapter primers and the resultant altered GJA1 sequences were cloned into pEYFP-N1. The following mutagenic forward primers and their complementary sequences as reverse primers were used: Y175, 5′-CCAAGTTCAACCTCTTCAACTGCG- TGGAGGGGAAG-3′; G21R, 5′-CTTACTCACTGCTAGGGAGGACTGGGCG- GTTCAG-3′; A40V, 5′-CTCCTGCTGGATTAGCTGTGGTGTTGACCTGTT-3′; F52dup, 5′-CCTGGGAGATGAGGCTGCTTTTTCGCTGTAACACTG- AGC-3′; G21R, 5′-CTTCCGGGAGATCTCCAGGT-CATCAGGCC-3′; and I130T, 5′-CAAGTGGCAGATCGACCTAGCGACACTGAAAG-3′.

Conditions of amplification were determined on a BioRad Laboratories iCycler (Hercules, CA). All amplifications were performed using Platinum Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA). Plasmid inserts were sequenced to confirm wild-type and altered GJA1 sequences by the dideoxy chain termination method on an ABI Prism 3700 automated fluorescent DNA analyzer (Applied Biosystems, Foster City, CA).

Subcloning of Cx43-eYFP constructs into BH-RCAS retroviral expression vector

Mutant Cx43-eYFP fusion constructs were amplified using the following adapter primers containing the underlined ClaI sites: forward 5′-GATCATATCTGACAG-CAGGCGATTT-3′ and reverse 5′-GATCATATCTGACAGGCGATTT-3′. PCR was performed using high fidelity Deep-Vent DNA polymerase (New England Biolabs, Beverly, MA). PCR products were digested with ClaI and ligated into ClaI-linearized BH-RCAS, a replication-competent retroviral vector derived from the Rous sarcoma virus (Hughes et al., 1990). Entire Cx43-coding sequences were again verified at UCLA Sequencing Core Facility using an ABI Prism 3700 analyzer.
Expression of Cx43-eYFP fusion constructs in DF-1 and production of recombinant viral particles

Transfection of DF-1 cells with BH-RCAS constructs encoding Cx43-eYFP was performed using Superfect (Qiagen, Valencia, CA) in 60 mm dishes according to manufacturer’s instructions with 5 µg plasmid DNA and 20 µl Superfect. Stable expression of Cx43-eYFP constructs after 10-14 days of routine passaging was achieved in the majority of cells with concomitant production and release of infectious recombinant viral particles containing Cx43-eYFP mutants into the supernatants. Infectious target cells with such viral particles resulted in stable expression of wild-type and mutant Cx43-eYFPs. DF-1 cells were used to produce recombinant virus; all functional studies described below were performed in C6 cells.

Expression of Cx43-eYFP constructs in C6 cells

To render C6 cells susceptible to infection with virus, C6 cells were transfected with pbCB6095), an expression plasmid encoding the 157 amino acid isoform Tva receptor (gift from Dr Paul Bates, University of Pennsylvania, Philadelphia, PA) using Superfect transfection reagent (Bates et al., 1993). Transfected C6 cells were selected in 600 µg ml⁻¹ G418. Clonal populations were screened for expression of Tva by indirect immunofluorescence using the rabbit anti-serum anti-Tva #40 (gift from Dr Paul Bates). One clone (C6R) was isolated, expanded and used for further experiments.

To isolate recombinant virus for infection of C6R cells, tissue culture medium from a confluent 10 cm dish of DF-1 expressing a Cx43-eYFP construct was replaced with 0.5 ml of 1 mg ml⁻¹ 4% paraformaldehyde in DPBS containing 0.5 ml of 1 mg ml⁻¹ PI. The cells were then incubated for 20 minutes and then rinsed three times with regular HBSS and once with 0 Ca²⁺ HBSS. The cell monolayer was then scraped from the tissue culture plate using a razor blade applied with downward force only and incubated at room temperature for 5-10 minutes. Although sulforhodamine B is a non-fixable dye, we found that these cells generally had significantly higher intensities. At least three independent experiments were performed on at least two clones per mutant.

Laser-scanning confocal microscopy of Cx43-eYFP in live C6 cells

A custom confocal microscope equipped with a blue diode laser (475 nm) and an inverted 60X 1.3 apochromat objective (Olympus, Melville, NY) was used. Cells were plated on uncoated glass coverslips and allowed to attach for 1-2 days before imaging. Live cells were imaged at room temperature in Hank’s Balanced Salt Solution (HBSS) (Mediatech). Images represented frame-averaging of roughly 150 frames acquired over 10 seconds.

Scrape-loading dye transfer assay

C6 cells were seeded on uncoated glass coverslips (18 mm) in 12-well plates at a density in which confluent monolayers will be achieved by 48-72 hours. For Ca²⁺ conditions, cells were washed twice with HBSS containing Ca²⁺ and incubated for 15 minutes in HBSS containing 0.5 ml of 1 mg ml⁻¹ PI (Pierce, Rockville, MD). PI was then aspirated and cells rinsed three times with regular HBSS, fixed with 4% paraformaldehyde and mounted on glass slides. Cells close to the center of the coverslip were selected using the 20X objective using rhodamine settings and photographed with a digital camera mounted on a Nikon upright microscope. Images are analyzed by ImageJ software. Regions of interest were drawn through 40-50 cells and average intensities calculated. Approximately 10% of control and mutant cells appeared to show non-specific uptake in 0 Ca²⁺ HBSS conditions and were excluded from quantitation.

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References


Function of ODDD-associated Cx43 mutants

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